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14. ABSTRACT The goals of the first year/phase of the DOD funded research, PC073499, have been accomplished. We produced two human versions of the OX40 ligand:trimer:Ig protein. The two protein constructs have different human trimer domains (TRAF2 and Matrilin-4), which confer differing folding patterns within the two-dimension structure of the protein. Both the TRAF2 and Matrilin-4 trimer domain constructs appear to have as good if not better in vitro bioactivity when compared to a mouse anti-human OX40 Ab that is currently in a clinical trial for cancer patient treatment. We have produced enough purified material to test the in vivo activity of these proteins and have planned a pilot study involving injection of these new human OX40 agonists in non-human primates. Upon completion of this project we will have important preclinical information that should allow for FDA approval of a fully human OX40 agonist to be tested in a clinical trial.				
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DOD Prostate Award Research Technical Reporting: 1 year Progress Report
Laboratory-Clinical Transition Award: Stage I

PC073499 – Production and Characterization of a Novel OX40 Ligand for Clinical Use.
PI -Andrew Weinberg, PhD

INTRODUCTION: Cancer cells have evolved to evade immune-mediated destruction through several documented mechanisms. Our group has developed a technique to enhance immune function in tumor-bearing hosts through the use of OX40 agonists, which can lead to regression of tumors of various histologies, including prostate cancer (1). In particular, we have produced a human OX40 agonist, termed OX40L:ILZ:Ig that has potent biologic function in vitro and is produced in large quantities by tissue culture cells. The ILZ portion of the chimeric protein was initially a trimerization domain obtained from a yeast sequence. Our initial goal is to fully humanize this molecule by incorporating human trimerization domains to replace the yeast sequence, thus lowering the probability of immune-mediated recognition of this recombinant protein by treated cancer patients. Once a fully functional human OX40 ligand protein is developed we will proceed with production and purification of the protein so that it can be tested for in vivo biologic activity in non-human primates. If the biologic activity is as potent as an OX40 agonist Ab that we currently have administered to patients we will produce enough GMP material to test in a formal toxicity study in non-human primates. This should give us the proper data to approach the FDA for approval of this drug for clinical trial use. Ultimately, the goal of the research is to produce clinical grade human OX40L:ILZ:Ig to test in clinical trials for patients suffering from prostate cancer.

BODY: The initial year of funding was spent on perfecting the soluble OX40 ligand molecule. In particular, we proposed to swap the yeast ILZ domain with known human trimerization domains. After modeling the different known human trimerization domains we made a decision to test two types: 1) a non-covalent trimerization domain (from TRAF2 sequence) and 2) a disulfide linked covalent trimerization domain (from matrilin-4 sequence). TRAF2 is an adapter protein that directly associates with the OX40

cytoplasmic tail and ultimately is involved with transmitting a downstream signal to the nucleus. It has been shown that OX40 and OX40 ligand form a trimer at the cell surface and the associated TRAF protein(s) form a trimer within the cytoplasm essentially forming a stacked three protein trimer complex. Hence we hypothesized that the TRAF2 trimer domain might make a perfect spatial fit between the OX40L extracellular domain and the Ig tail. There are only a few of the human trimerization domains that form natural disulfide bonds and the matrilin-4 protein is one of them. We reasoned that a covalently bonded trimerization domain might offer greater stability in vivo and hence might have increased biologic activity when compared to a non-covalent trimer domain. Therefore we produced both OX40L constructs and expressed them in 293 cells as secreted proteins. Protein G column chromatography was used to purify the proteins to >95% homogeneity and we compared their size by native gel electrophoresis. Prior to these experiments we published (2) that our initial recombinant protein, OX40L:ILZ:Ig, containing the yeast trimerization domain folded predominantly as a hexamer as determined by column chromatography. Therefore we ran all three recombinant proteins side by side on the native gels to get an idea of heterogeneity and comparative molecular weights (Figure 1). It was clear that the TRAF2 and matrilin-4 trimerization domains gave similar protein products (3 bands) but the matrilin-4 protein was less homogenous compared to the TRAF2 construct. We surmised that the upper band corresponded to a dodecamer, the middle band a hexamer, and the lower band a trimer. The matrilin-4 domain clearly had more of the higher and lower MW bands, which may or may not correspond to increased biologic activity. At this point we tend to favor the TRAF2 construct due to the more homogenous nature of the product, although we are now in the process of testing all the protein products in our in vitro T cell stimulation assay. Figure 2 shows some of our initial bioassay results and it is clear that both protein constructs have potent biologic activity, both in the plate-bound and soluble assays. While it is hard to pick a clear cut “winner” based on our in vitro assays we plan to test both of these constructs in a non-human primate pilot study.

Initially within our Statement of Work document we did not include the small pilot study discussed above, but we now feel that in vivo verification of our fully humanized ligand(s) is imperative and prudent prior to moving forward with large-scale

protein production and a full toxicity study proposed in years 2 and 3. The pilot project will also help us make a decision as to which construct we should choose as we go forward when making clinical trial material. To this end we have produced 100 mg of both the TRAF2 and Matrilin-4/OX40L chimeric proteins and shown that this material contains very low levels of endotoxin. Hence, we are ready to proceed with the monkey studies and have obtained IACUC approval from the Oregon Primate Center and we are currently seeking approval from the DOD to proceed with the pilot study. It should be noted that the human OX40 ligand only binds to human and monkey (not mouse or rat), therefore we need to test our protein constructs in monkeys prior to moving forward.

KEY RESEARCH ACCOMPLISHMENTS:

- We produced two novel forms of the human OX40 ligand:trimer:Ig protein that are now complete human sequences.
- These proteins were translated efficiently in 293 cells and easily purified by protein G chromatography.
- It is clear that the TRAF2 trimer domain and the Matrilin-4 trimer domain confer differences in protein size ratios, which was ascertained by native gels.
- Both humanized OX40 ligand constructs have potent biologic activity as assessed by an in vitro T cell costimulation assay.
- We have purified 100 mg quantities of the two humanized OX40 ligand proteins to be used in an in vivo pilot study in non-human primates.

REPORTABLE OUTCOMES:

We have yet to publish these results as we have just produced these proteins and have only performed two in vitro costimulation assays (Figure 2). Our hope is that the in vivo biologic activity may represent a reportable outcome in year 2 of the funded work.

CONCLUSION: In summary, we have completed the first year/phase of the research, which was to produce a fully human version of the OX40 ligand:trimer:Ig protein. We produced two protein constructs with two different human trimer domains and both had biologic activity as assessed by our in vitro T cell costimulation assay. We have

produced enough purified material to test in vivo activity and have planned a pilot study involving injection of these proteins into non-human primates. This pilot study was not in the original statement of work, but we are currently in process of submitting a revised statement and budget to support this in vivo study. Both the TRAF2 and Matrilin-4 constructs appear to have as good if not better in vitro bioactivity when compared to a mouse anti-human OX40 Ab that we currently have in clinical trials. In our current phase I clinical trial with a mouse OX40 agonist Ab that is administered to stage IV cancer patients we have observed some tumor shrinkage in the first 5/20 patients, however we have not yet observed a complete response. Our hope is that the fully human OX40 agonists described herein will have more potent biologic activity. Ultimately, upon completion of this project we will have important preclinical information that should allow for FDA approval of a fully human OX40 agonist.

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2. Morris NP, Peters C, Montler R, Hu HM, Curti BD, Urban WJ, **Weinberg AD**. Development and Characterization of Recombinant Human Fc: OX40L fusion protein linked via a coiled-coil trimerization domain. Molecular Immunol. 44:3112-3121. 2007.

APPENDICES: Not applicable

SUPPORTING DATA:

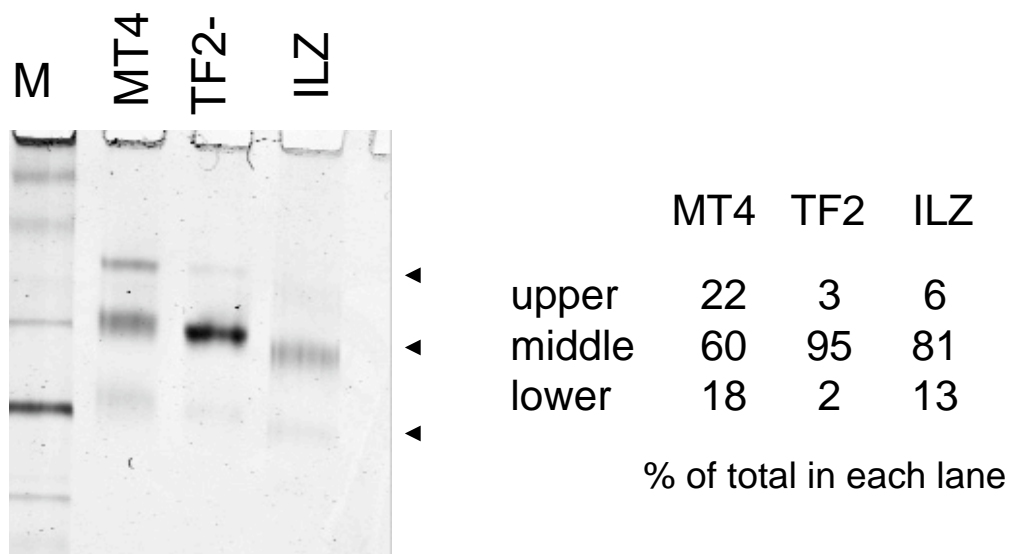


Figure 1. Analysis of recombinant human soluble OX40L constructs by native polyacrylamide gel electrophoresis. Soluble OX40L constructs contain (from N-terminus to C-terminus) the Fc γ domain of human IgG1, the coiled coil domain for either yeast GCN4 (ILZ), human matrilin 4 (MT4) or human Traf2 (TF2) and the extracellular domain of human OX40L. Aliquots of these three proteins along with marker (M) were analyzed by native gel electrophoresis using the the coomassie blue method (Invitrogen). Separation is based on mass and shape as in size exclusion chromatography but with high resolution. The relative proportion of each band in the three samples was quantified on an Odyssey Infrared scanner (Licor) and shown to the right of the gel.

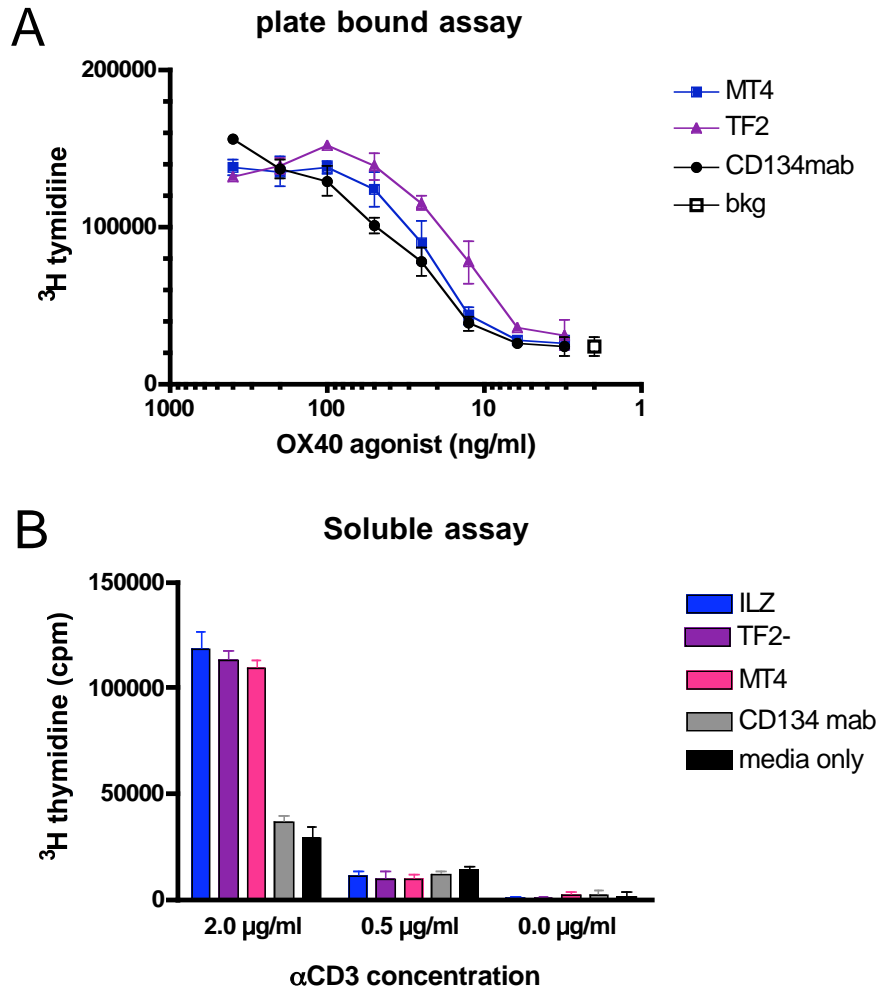


Figure 2. Functional comparison of soluble human OX40L constructs.

The activity of two fully human OX40L constructs, Fc-Traf2(TF2)-OX40L and Fc-Matrilin-4 (MT4)-OX40L were compared human-yeast hybrid construct Fc-GCN4(ILZ)-OX40L and to the mouse anti-human OX40 antibody, CD134 mab (clone 9B12), currently in phase I clinical trial. The assay is based on the ability of OX40 agonists to stimulate human CD4 T cell proliferation, as measured by incorporation of ^3H -thymidine. **A.** In the plate bound assay, the activity of the plate bound OX40 agonist is measured in the presence of plate-bound sub-threshold anti-CD3 stimulation of the T cell receptor complex. **B.** In the soluble assay, activity of soluble OX40 agonist (10 $\mu\text{g/ml}$) is measured in the presence of sub-threshold anti-CD3 TCR stimulation.